

REMARKS/ARGUMENTS

Claims 3-9 and 25-29 are presently pending. Entry of the foregoing and favorable reconsideration and reexamination of the subject application, as amended, pursuant to and consistent with 37 C.F.R. Section 1.112, and in light of the remarks which follow, are respectfully requested.

The rejection of Claims 4, 7, 9 and 25-27 under 35 U.S.C. § 112, first paragraph (enablement) is respectfully traversed.

In rendering this rejection, the Examiner purports that it would be undue experimentation to obtain a vaccine for malaria since a vaccine for malaria has not yet been successfully achieved and there is a lack of guidance in the specification for the claimed vaccine compositions. Applicant respectfully disagrees with the Examiner's conclusion for the following reasons.

The Examiner has cited several references in support of the above conclusion, which references will be discussed in more detail below. The only conclusion that can be drawn from these references is that *they are not relevant to the subject matter of the claimed invention*.

More specifically, the Examiner relies on Arevalo-Herrera et al and concludes the complexity of the parasite's life cycle makes a universal, effective and long-lasting vaccine difficult to produce. The entire article is based on *P. vivax* vaccines of the pre-erythrocyte stage, the CS protein, the sporozoite surface protein 2, the asexual blood stages, the duffy binding protein and the apical membrane antigen 1. With respect to MSP-3 antigen, the authors merely state that it is being characterized and currently being analyzed for antigenicity and epitope mapping. Thus, it cannot be said that the problems associated with

malaria vaccines as described in Arevalo-Herrera et al would apply to MSP-3, since no information was available concerning the vaccine potential of this antigen at the publication date of this reference.

Bouharoun-Tayoun et al is a scientific article that reports that the Hu-SPL-SCID model can be successfully employed to induce human antibody-secreting cells specific of low immunogenicity neo-antigens such as MSP-3, which represent a new and valuable source of human monoclonal anti-malaria antibodies. The only mention of a vaccine in this article is on page 2, which describes MSP-3 as a vaccine candidate, but does not describe any results or why MSP-3 qualifies as a vaccine candidate.

Kurtis et al is directed to the LSA-1 antigen, which is a liver stage antigen and is not a merozoite stage antigen. The conclusions of this article are therefore irrelevant to the presently claimed invention.

Shi et al describe a multistage and multivalent vaccine, which would probably be efficacious (See, Abstract). Table 1 describes the amino acid sequences, as well as the antigen-epitope that were used in the development of the vaccine. MSP-3 is not described among the antigen-epitopes used in their vaccines. Therefore, this reference is also irrelevant with respect to the claimed invention.

It appears that the Examiner, in rendering this rejection, is misinterpreting the claims to recite a commercialized vaccine. However, the claims solely recite a vaccine. The sole criteria of a vaccine is to stimulate an immune response that can prevent an infection or create resistance to an infection or to reduce the parasite load or reduce or eliminate parasite replication or growth after invasion. T-cell and antibody responses are indicative of an immune response. Applicant submits that the present invention shows such responses.

Indeed, the present specification clearly shows the results of a first clinical trial undertaken using the various claimed peptides. This clinical trial was performed on 36 human subjects, which were administered the MSP-3 peptides of the present invention. The results from these clinical trials were as follows:

- (1) Antibody and T-cell responses remained detectable at month-4 and were boostable by the third immunization;
- (2) IFN-gamma secretion was extremely high; 10,000 to 50,000 International Units;
- (3) Subclasses of IgG1 and IgG3 predominated over IgG2 and IgG4, which is the optimal profile for isotype distribution;
- (4) Over all immunogenicity was much higher than recorded in pre-clinical models of mice and South American primates where responses were lower with Montanide® and absent with alum;
- (5) The functional bioassay results demonstrated that the vaccine containing the peptides of the present invention is safe, immunogenic, induces the classes of IgG needed to have a biological effect and that the antibodies have the ability, in cooperation with monocytes, to exert a killing effect upon *P. falciparum*.

The results set forth in the examples of the present invention are clearly indicative that the peptides of the presently claimed invention, *in fact*, produce an immune response indicative of a vaccine.

Finally it can be said that further studies carried out using several of the claimed peptides of the present invention confirmed the important epitopes of MSP-3b, MSP-3c and MSP-3d fragments as set forth in Singh et al (see Annex II, submitted herewith). Singh et al demonstrated that high IgG titers against MSP-3b, MSP-3c and MSP-3d peptides were

observed among protected, compared with non-protected human subjects, as indicated at page 1015, first column, second paragraph.

Moreover, in Meraldi et al (see Annex III, **submitted herewith**) a longitudinal study was undertaken in Burkina Faso among 293 children aged 6 months to 9 years. The conclusion reached in this study was that children had a positive association between their level of IgG specific for MSP-3 154-249 before the raining season and subsequent protection from clinical malaria episodes.

Both Singh et al and Meraldi et al clearly demonstrate the success of the presently claimed invention in human studies. Thus, Applicants submit that the presently claimed invention is, *in fact*, enabled. As such, withdrawal of this rejection is respectfully requested.

The rejection of Claims 3-7 and 25-27 under 35 U.S.C. § 102 (b) over McColl et al is respectfully traversed.

McColl et al disclose the complete MSP-3 amino acid sequence from clones D10 and 3D7 and isolates K1 and CSL-2, having 380, 354, 379 and 379 amino acids in length, respectively. McColl et al, however, do not disclose SEQ ID No. 11 to 14 as immunogenic compositions or vaccines, which are shorter peptide sequences.

Indeed, McColl et al emphasizes that the heptad repeats illustrated in the boxes in Figure 1 at page 25 would be likely candidates to possess immunodominant epitopes. However, none of the claimed sequences of the present invention are exclusively present in the boxes of Figure 1.

Since the claimed peptide sequences of the present invention are not disclosed in this reference, Applicant submits that the presently claimed invention is novel over McColl et al.

In view of the foregoing, withdrawal of this rejection is respectfully requested.

The rejections of Claims 3-7 and 25-27 under 35 U.S.C. § 102 (b) over Oeuvray et al Blood 1994 or Oeuvray et al 1994 (Mem. Inst. Oswaldo Cruz, Rio de Janeiro, Supp. 11) are respectfully traversed.

It should be noted in that neither reference of Oeuvray et al discloses SEQ ID Nos. 11 to 14, as the Examiner maintains. Only SEQ ID No. 12 was identified in these references and hence this particular sequence is no longer recited as a sole sequence in the immunogenic composition. Moreover, neither reference of Oeuvray et al discloses an immunogenic composition that has a combination of MSP-3b, MSP-3c and MSP-3d, as now recited in new Claims 28 and 29.

Furthermore, a close evaluation of the MSP-3a sequences in Oeuvray et al, Blood 1994 and Oeuvray et al, 1994, reveals that this sequence starts with the amino acid His (H), while the sequence of the MSP-3a of the present invention starts with a Tyr (Y).

Likewise, the MSP-3 c peptide identified in both Oeuvray et al references ends with the amino acid Glu (E). In contrast in the present invention, the MSP-3c peptide ends with the amino acid Asp (D).

Finally, an MSP3d sequence is not identified in either reference of Oeuvray et al.

Thus, the presently claimed invention is not anticipated by Oeuvray et al Blood 1994 or Oeuvray 1994, since the claimed sequences are not disclosed in these cited prior art documents.

Therefore, in view of the above, withdrawal of this rejection is respectfully requested.

The rejection of Claims 3 and 8 under 35 U.S.C. § 103 (a) over McColl et al taken with Saul et al is respectfully traversed.

McColl et al do not disclose the specifically recited MSP-3 peptides of SEQ ID NOs. 13 and 14. Indeed, McColl et al disclose sequences having a length of 380, 354 and 379 amino acids of MSP-3 polypeptides from clones D10 and 3D7 and isolates K1 and CSL-2. McColl et al do not disclose or even suggest using any shorter peptides for vaccination purposes.

The secondary reference of Saul et al do not remedy the deficiencies of the primary reference, since the specifically claimed MSP-3 sequences are not disclosed nor suggested in Saul et al. Indeed, the peptides used in Saul et al are a full length MSP-2 peptide, a 175 amino acid fragment of an MSP-1 peptide and a C-terminal 70% RESA peptide. There is simply no disclosure or teaching to motivate the person skilled in the art to use shorter MSP-3 sequences, let alone those which are currently claimed.

Since neither reference discloses nor suggests the MSP-3 peptides of the present invention, Applicant submits that this rejection cannot be maintained.

Thus, in view of the above, withdrawal of this rejection is respectfully requested.

The rejection of Claims 7-9 and 27 under 35 U.S.C. § 112, second paragraph, and the objection to the specification for including tradenames are obviated by amendment.

More specifically, the following amendments have been made, which are believed to adequately address the Examiner's criticisms:

- Claim 7 has been amended to delete the phrase "or the vaccine;"
- Claims 8 and 9 have been amended to replace "Montanide" with the generic description of Montanide®;
- Claims 8 and 9 have been amended to remove the conjunctive term "and;" and
- Claim 27 has been amended to delete "immunogenic composition or."

Applicants have also amended the specification to indicate that “Montanide®” is a trademark and the generic terminology has been inserted for this trademark. Montanide® has also been capitalized wherever it appears. Support for the generic terminology for “Montanide®” is provided by Hanly et al (see, Annex I **submitted herewith**).

In view of the foregoing, withdrawal of the indefiniteness rejection and the objection to the specification is respectfully requested.

By the present amendment, the specification has been amended to indicate that “Montanide®” is a trademark and the generic terminology has been inserted for this trademark. Montanide® has also been capitalized wherever it appears.

Claims 3, 5, 7 to 9, 25 and 27 have been amended to further clarify the present invention or to insert the generic terminology of the trademark Montanide® into the claims (See, Annex I). Claims 28 and 29 have been added. Applicant submits that no new matter has been added via this amendment.

The objection to the Abstract as not adequately describing the claimed invention is believed to be obviated by the substitute Abstract **submitted herewith**. Applicant request withdrawal of this ground of objection.

Finally, consistent with the Examiner’s request, the status of the related applications appearing in the continuity data of the present application has been updated.

Applicants submit that the present application is now in condition for allowance.

Early notification of such action is earnestly solicited.

Respectfully submitted,

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ANNEX I

<http://www.nal.usda.gov/awic/pubs/antibody/overview.htm>

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The following was adapted from a series of short articles published in the August and September 1994 issues of the BRL Bulletin. These articles were intended to explain briefly the function of adjuvants; guidelines for use of adjuvants, particularly Freund's adjuvants; and to introduce alternative adjuvants. For a more in depth discussion of adjuvants and antibody production patrons are referred to the ILAR Journal, volume 37; number 3, 1995.

Montanide ISA Adjuvants [Seppic, Paris, France] are a group of oil/surfactant based adjuvants in which different surfactants are combined with either a non-metabolizable mineral oil, a metabolizable oil, or a mixture of the two. They are prepared for use as an emulsion with aqueous Ag solution. The surfactant for Montanide ISA 50 [ISA = Incomplete Seppic Adjuvant] is mannide oleate, a major component of the surfactant in Freund's adjuvants. The surfactants of the Montanide group undergo strict quality control to guard against contamination by any substances that could cause excessive inflammation, as has been found for some lots of Arlacel A used in Freund's adjuvant. The various Montanide ISA group of adjuvants are used as water-in-oil emulsions, oil-in-water emulsions, or water-in-oil-in-water emulsions. The different adjuvants accommodate different aqueous phase/oil phase ratios, because of the variety of surfactant and oil combinations. The performance of these adjuvants is said to be similar to Incomplete Freund's Adjuvant [IFA] for antibody production; however the inflammatory response is usually less.

ANNEX II

MAJOR ARTICLE

Identification of a Conserved Region of *Plasmodium falciparum* MSP3 Targeted by Biologically Active Antibodies to Improve Vaccine Design

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Merozoite surface protein 3 (MSP3) is a target of antibody-dependent cellular inhibition (ADCI); a protective mechanism against *Plasmodium falciparum* malaria. From the C-terminal half of the molecule, 6 overlapping peptides were chosen to characterize human immune responses. Each peptide defined at least 1 non-cross-reactive B cell epitope. Distinct patterns of antibody responses, by level and IgG subclass distribution, were observed in inhabitants of a malaria-endemic area. Antibodies affinity purified toward each peptide differed in their functional capacity to mediate parasite killing in ADCI assays: 3 of 6 overlapping peptides had a major inhibitory effect on parasite growth. This result was confirmed by the passive transfer of anti-MSP3 antibodies in vivo in a *P. falciparum* mouse model. T helper cell epitopes were identified in each peptide. Antigenicity and functional assays identified a 70-amino acid conserved domain of MSP3 as a target of biologically active antibodies to be included in future vaccine constructs based on MSP3.

The asexual blood-stage multiplication of the malarial parasite is responsible for the acute symptoms of malaria in humans. Epidemiological observations have shown that adults living in endemic areas, although they are constantly reinfected and frequently carry parasites, control their levels of parasitemia and show substantial clinical resistance, compared with children [1]. Repeated infections and continued exposure to the parasite are required to reach this level of immunity against disease [2]. This state of naturally acquired immunity against disease, a phenomenon that is called premunition [3], is not a sterile immunity and is marked by chronic low-grade parasitemia without clinical symptoms.

The passive transfer of serum IgG from clinically immune individuals has been shown to be able to control disease and the level of parasitemia in nonprotect-

ed individuals who are exposed to geographically diverse parasite strains [4–6]. We have earlier shown that the protection afforded by IgG has no major direct effect on parasite invasion and growth in red blood cells (RBCs)—rather, it acts in association with blood monocytes through an antibody-dependent cellular inhibition (ADCI) mechanism that inhibits parasite development [7]. The cytophilic nature of protective IgG has been established [8, 9], and the importance of these antibodies in protection against malaria has also been demonstrated in other independent studies [10, 11].

Our search for the targets of the protective antibodies, using ADCI as a functional assay, led us to identify merozoite surface protein 3 (MSP3) as one such target [12]. MSP3 is associated with merozoite surface molecules, possibly through the coiled-coil structures that have been predicted to be formed by the heptad repeats and the C-terminal leucine zipper domain [13]. The N-terminal part of the molecule consists of regions that are polymorphic among different strains. In contrast, the C-terminal part of the molecule is highly conserved among the various isolates of the parasite [14, 15], and it is this region that was earlier identified by screening of a *Plasmodium falciparum* expression library by use

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of functional ADCI assays [12]. Previous studies of MSP3 have focused only on a 27-aa region (aa 184–210, corresponding to the 3D7 strain, MSP3b) of the C-terminal part, which was earlier identified as a target of protective antibody response in hyperimmune serum samples [12].

We decided to further characterize the antigenicity of other regions in the C-terminal part of the molecule. Six overlapping peptides were designed (MSP3a, MSP3b, MSP3c, MSP3d, MSP3e, and MSP3f), each of which represented a different region of the conserved C-terminal part of the molecule. They were used to analyze the naturally occurring immune responses in individuals from the malaria-endemic village of Dielmo, Senegal, and their potential relationship to protection from malaria disease. The functional role of human antibodies specific to each region was assessed under *in vitro* conditions in the ADCI assay and was further confirmed by passive transfer *in vivo* in an immunodeficient mouse model grafted with *P. falciparum*-infected human RBCs [16, 17].

This process led us to identify a 70-aa region of MSP3 as the target for naturally occurring protective antibody responses. This region thus defines the minimal domain essential for the design of any vaccine construct based on MSP3.

MATERIALS AND METHODS

Antigens. MSP3 recombinant protein constructs and peptides were designed on the basis of the *P. falciparum* 3D7 strain sequence (NCBI protein_id, NP_700818.1). Two recombinant hexahistidine-tagged proteins, MSP3-NTH₁₋₁₁₄ and MSP3-CTH₁₈₁₋₃₀₀, were purified as described elsewhere [18]. The 6 peptides (MSP3a₁₆₇₋₁₉₁, MSP3b₁₉₁₋₂₁₀, MSP3c₂₁₀₋₂₃₀, MSP3d₂₁₁₋₂₃₀, MSP3e₂₃₀₋₂₅₀, and MSP3f₂₅₀₋₃₃₄) correspond to the conserved region of MSP3 C-terminal region. A small region (aa 253–274; 72% glutamic acid) was excluded from this analysis because glutamate-rich antigenic determinants exhibit cross-reactivity among several different *P. falciparum* antigens [19]. The peptides were synthesized according to standard peptide synthesis procedures [20].

Human serum and lymphocyte samples. For the affinity purification of antibodies specific to each MSP3 region, we used serum samples from 30 hyperimmune individuals from Ivory Coast that had been previously used for passive-transfer experiments in Thai patients with malaria and were found to be effective in controlling disease and parasitemia [6].

For immunoepidemiological studies, we used plasma samples from 48 permanent residents of the village of Dielmo, Senegal, who had various degrees of exposure to malaria (age, 3.5–53.4 years; mean age, 13.1 ± 1.8 years; mean stay in the village, 707/730 days of follow-up). In this region, malaria transmission is intense and perennial (~200 infected mosquito bites/person/year); over the course of 2-year period, the mean number of malaria attacks was 2.4 ± 5.4 episodes/person. Nine-

teen individuals had no malaria attack (mean age, 15.7 ± 3.1 years), whereas 29 individuals had at least 1 malaria attack (mean age, 11.4 ± 2.2 years) during the next 2 years. All inhabitants of Dielmo were actively monitored by medical doctors on a daily basis for febrile episodes, and those due to malaria were accurately diagnosed as described elsewhere [21]. This allowed us to examine the pattern of the IgG isotype response toward different regions of MSP3 in individuals who were clearly distinguishable as “protected” (no malaria attack) or “nonprotected” (≥1 malaria attack) during the 2-year follow-up period of the study. This group was representative of the whole village in terms of age distribution, with respect to occurrence or absence of malaria attack.

Mononuclear cells obtained from inhabitants of Dielmo were transported within 4 h to laboratories in Dakar and used for T cell proliferation and the determination of interferon (IFN)- γ against MSP3a, MSP3b, and MSP3c peptides, according to methods described elsewhere [22, 23]. In brief, the proliferative responses of the cells were assessed in quadruplicate in 96-well round-bottomed plates (Nunc; Nunc) by incubation for 6 days at 37°C in 5% CO₂ in the presence of each peptide used at 10 μ g/mL, followed by the addition of 1 μ Ci of [³H]thymidine overnight and counting of the incorporated radioactivity in a liquid scintillation counter. Unstimulated cultures served as negative controls, and purified protein derivative and phytohemagglutinin were used as positive controls. The IFN- γ concentration in pooled supernatants from quadruplicate wells was assessed by a capture ELISA performed in duplicate, by use of the anti-human IFN- γ monoclonal antibody (MAb) 350B10G6 and biotin-labeled MAb 67F12A8 (Biosource) for coating and revealing, respectively, according to the manufacturer's instructions. The reaction was revealed by use of streptavidin-horseradish peroxidase and tetra-methyl benzidine chromogen, and the optical density was measured at 450 nm. For practical reasons, mainly the number of cells available per donor, the other 3 peptides used for antibody assays could not be included in T cell assays. Lymphoproliferation studies were performed with samples from 61 inhabitants (29 female and 32 male; mean age, 27.31 years), and IFN- γ secretion was studied in 31 inhabitants (19 female and 12 male; mean age, 33.94 years). The 3 peptides proved to induce no significant response in peripheral blood mononuclear cells from 16 control, non-malaria-exposed donors (data not shown), which indicated that they had no mitogenic or superantigenic effect.

ELISA. The ELISA was performed for the detection of total IgG and subclasses, as described elsewhere [8, 9]. Monoclonal mouse anti-human subclasses IgG1–IgG4 (clones NL16 [Boehringer], HP6002 [Sigma], Zg4 [Immunotech], and RJ4 [Immunotech]) were selected for their affinity and reactivity for African allotypes and were used as secondary antibodies at dilutions of 1:2000, 1:5000, 1:5000, and 1:1000, respectively.

The specific reactivity of each serum sample was obtained by subtracting the optical density value of a control protein (0.25 μ g of bovine serum albumin/well) from that of the test antigens. For calculating the threshold of significance of antibody responses, a set of 8 randomly selected serum samples from individuals never exposed to malaria was tested against each antigen, as controls. Results were expressed as the ratio of the mean optical density from test serum samples to the mean optical density of control subset $\pm 3 \times$ the SD of the control serum samples. Serum samples were considered to be positive for ratios ≥ 1 .

Affinity purification of antibodies. Because the ADCI assay requires the cooperation of antibodies with the Fc- γ RII receptor [7], a group of 30 hyperimmune serum samples from individuals from Ivory Coast were first screened for IgG subclass distribution against different MSP3 peptides and recombinants. Serum samples were selected for the affinity purification of antibodies against any given MSP3 construct on the basis of their high reactivity against that region, with minimal reactivity toward the adjacent peptides and a high content of cytophilic IgG antibodies (IgG1 and IgG3). Independent serum pools (each of which was made up of 3–7 individual serum samples) were used to affinity purify antibodies to different regions of MSP3. The ratios of cytophilic to noncytophilic IgG subclasses (IgG1 + IgG3:IgG2 + IgG4) of the serum pools used were estimated to be 9.56 for MSP3NT, 4.25 for MSP3CT, 1.29 for MSP3a, 3.86 for MSP3b, 1.29 for MSP3c, 4.58 for MSP3d, 1.59 for MSP3e, and 3.68 for MSP3f. Previous studies have shown that the profile of cytophilic antibodies observed in affinity-purified antibodies was similar to that of the serum sample pool used for affinity purification (S. Singh, S. Soe, and P.D., unpublished data).

Affinity purification was done as described elsewhere [24], by use of a 2.5% aqueous suspension of polystyrene beads (mean diameter, 10 μ m; Polysciences) to coat the peptides or recombinant proteins. Specific antibodies were eluted by use of 0.2 mol glycine/L (pH 2.5) and were immediately neutralized to pH 7.0 by use of a 2 mol/L aqueous Tris solution. Affinity-purified antibodies were dialyzed extensively against PBS followed by RPMI and were concentrated by use of Centricon concentrators (Millipore), filter sterilized, and, after the addition of 1% albumax (Gibco BRL), stored at 4°C. Affinity-purified antibodies were used at a concentration of 10 μ g/mL in ELISA to ascertain their specificity.

Immunofluorescence assay (IFA). Because the ability of the antibodies to recognize the native parasite protein is the critical factor in biological assays, IFA was used to adjust the concentration of affinity-purified antibodies. IFA was performed on air-dried, acetone-fixed, thin smears of *P. falciparum* mature schizonts, as described elsewhere [25], to assess the

binding activity of affinity-purified antibodies to the parasite protein. The effective concentration of each antibody was adjusted to a 1:200 IFA end-point titer for use in functional assays.

Functional in vitro antibody assays. The antibody-dependent, monocyte-mediated ADCI assays were performed in duplicate by use of laboratory-maintained strains 3D7 and Uganda Palo-Alto, as described elsewhere [7]. Monocytes from healthy, non-malaria-exposed donors were prepared as described elsewhere [7]. The affinity-purified antibodies, adjusted to a concentration yielding a 1/200 IFA end-point titer, were added at a rate of 10 μ L in 90 μ L of complete culture medium, which yielded a final titer of 1/20 in the ADCI assay. After cultivation for 96 h, the level of parasitemia was determined on Giemsa-stained thin smears from each well by the microscopic examination of $\geq 50,000$ erythrocytes. Monocyte-dependent parasite inhibition is expressed as the specific growth inhibition index (SGI): $SGI = 1 - ((\text{percentage of parasitemia with monocytes and test IgG}) / (\text{percentage of parasitemia with test IgG}) / (\text{percentage of parasitemia with monocytes and normal IgG}) / (\text{percentage of parasitemia with normal IgG})) \times 100$. Although the SGI calculation takes into account a possible direct antiparasite effect of monocytes, because this is observed with 10%–15% of monocyte preparations, we excluded them as an additional precaution.

Passive immunization of *P. falciparum*-infected immunocompromised mice. The use of the *P. falciparum*-human RBC (HuRBC)-Beige-Xid-Nude (BXN) mouse model for assessing the effect of antibodies on different blood-stage antigens of *P. falciparum* has been detailed elsewhere [16]. In brief, 6–8-week-old male BXN mice (Charles River Laboratories), manipulated under pathogen-free conditions, were treated with liposomes that contained dichloromethylene diphosphonate (Roche Diagnostics) and antipolymorphonuclear neutrophil Mab NIMP-R14 (NIMR), to reduce their innate immune response. *P. falciparum*-infected human RBCs were injected intraperitoneally (ip) on day 0, and uninfected RBCs were injected at 4-day intervals. The level of blood parasitemia was examined microscopically. Mice with stable parasitemia (0.1%–1%) were injected ip with 3×10^6 human peripheral blood monocytes positively selected by CD14⁺ magnetic beads (MACS; Miltenyi Biotech), followed 24 h later by the injection of 3×10^6 monocytes together with 200 μ L of affinity-purified antibodies to MSP3 at a 1:200 IFA end-point titer, as described above. Nonspecific esterase staining [7] showed that $>98\%$ of CD14⁺ cells were monocytes.

Statistical analysis. Univariate analysis was performed by use of the Mann-Whitney *U* test. Fisher's exact test was used for the contingency analysis. The association between the risk of malaria attack and the level of antibodies was tested with JMP software (SAS Institute), by use of a stepwise regression model in which we controlled for the confounding effect of

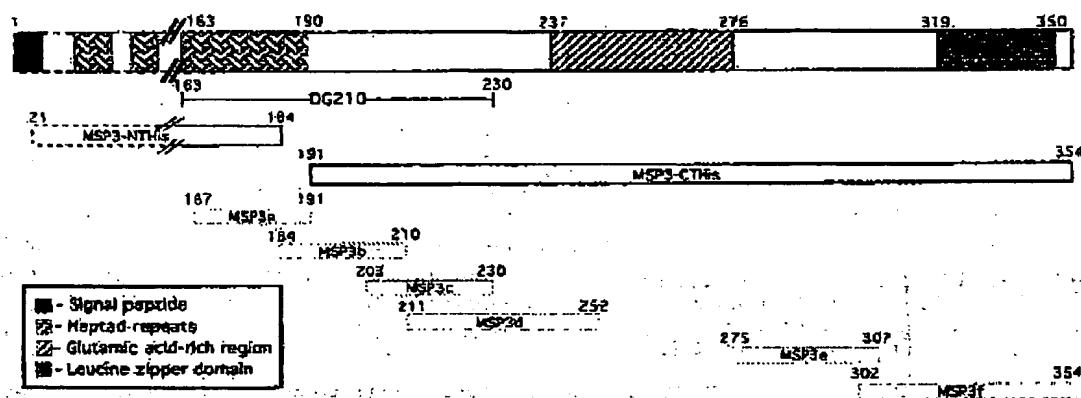


Figure 1. Schematic presentation of *Plasmodium falciparum* merozoite surface protein 3 (MSP3) and the design of MSP3 recombinant proteins (MSP3-NTHis and MSP3-CTHis) and peptides (MSP3a, MSP3b, MSP3c, MSP3d, MSP3e, and MSP3f). The representation of the N-terminal part of MSP3 is compressed here (dotted line). DG210 represents the λ gt11 expression clone originally identified as the target of protective antibodies [8]. The nos. show amino-acid positions for each region on the basis of the sequence derived from 3D7 strain.

age. The analysis of variance was applied to the regression model. The test of the null hypothesis was based on the variance ratio denoted by F , and departures from the null hypothesis tended to give values of F that were greater than unity.

RESULTS

Non-cross-reactive B cell epitopes defined by each of the 6 MSP3 C-terminal peptides. IgG responses were measured against

different regions of the MSP3 C-terminal (figure 1) in a group of 30 hyperimmune serum samples from individuals from Ivory Coast. As shown in figure 2, there were differences in the levels and prevalence of IgG toward each region, but antibody responses were detected against each of the C-terminal peptides.

Antibodies were then affinity purified from selected hyperimmune serum samples specific to each peptide and examined for their reactivity against the other peptides. In this way, it

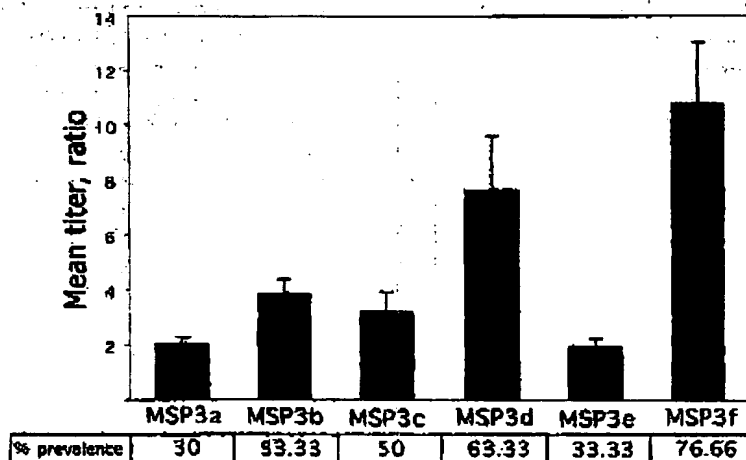


Figure 2. Total IgG response against different regions of merozoite surface protein 3 (MSP3) in hyperimmune serum samples ($n = 30$) from individuals from Ivory Coast, used to prepare protective IgG for passive-transfer experiments in humans [6]. Antibody reactivity was considered to be positive if the ratio of the mean optical density of the test serum samples to the mean optical density of control serum samples $\pm 3 \times$ the SD of the control serum sample was ≥ 1 . The figure represents the mean antibody titer (expressed as ratio) of positive serum samples against each region. The table shows the percentage prevalence of positive serum samples reactive to different regions of MSP3, in terms of total IgG.

Table 1. Specificity of affinity-purified human anti-merozoite surface protein 3 (MSP3) antibodies, as determined by ELISA.

Antibody	MSP3a	MSP3b	MSP3c	MSP3d	MSP3e	MSP3f
Anti-MSP3a	0.78	0.09	0.08	0.08	0.09	0.08
Anti-MSP3b	0.05	1.11	0.09	0.08	0.09	0.07
Anti-MSP3c	0.07	0.10	1.04	0.09	0.09	0.08
Anti-MSP3d	0.10	0.08	0.16	1.01	0.08	0.08
Anti-MSP3e	0.08	0.08	0.08	0.08	0.95	0.10
Anti-MSP3f	0.07	0.07	0.08	0.08	0.10	0.92

NOTE. Mean optical density values at 490 nm from duplicate wells are shown. All the peptides were used under identical coating conditions. Bold type represents positive reactivity.

was possible to affinity purify antibodies that were specific to each peptide but did not show cross-reactivity with other regions (table 1). These observations indicated that each of the peptides covering the MSP3 C terminal defines at least 1 B cell epitope that does not share antigenic determinants with other

regions. Each of the affinity-purified antibodies was also found to be positive in IFAs of *P. falciparum* asexual blood stages, which indicates that anti-peptide antibodies were reactive with the native parasite protein (data not shown).

Distinct isotype patterns of the IgG response toward different MSP3 peptides. We analyzed plasma from 48 individuals, 3–53 years old, from the endemic village of Dielmo, Senegal, to study the distribution and pattern of IgG isotype response against the different regions of the C-terminal part of MSP3 defined by the peptides. As shown in figure 3, both the level of antibody response and the pattern of IgG isotype were distinct against each region. The prevalence of responders varied for each region of MSP3 (6.25%–60.41% for IgG1, 4.16%–47.91% for IgG3, 0%–10.41% for IgG2, and 0%–12.5% for IgG4). We found that antibodies to MSP3a and MSP3e were less prevalent, and, when they were present, they were detected only at low levels. Antibodies to MSP3b, MSP3c, MSP3d, and

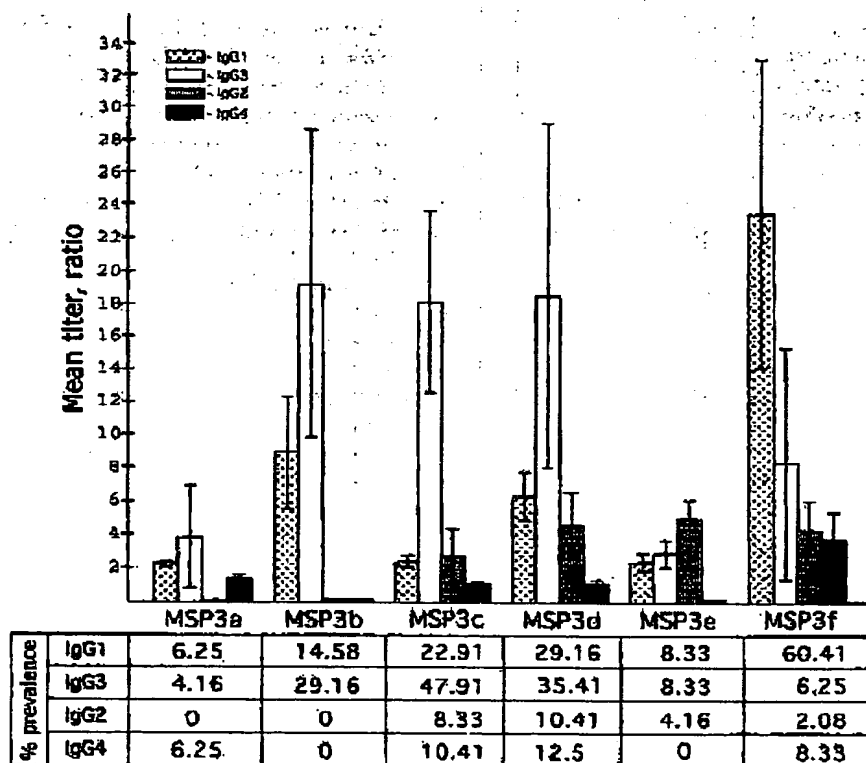


Figure 3. Prevalence and mean titer of antibodies against different regions of merozoite surface protein 3 (MSP3) in serum samples ($n = 48$) from Dielmo, Senegal. Antibody reactivity was considered to be positive if the ratio of the mean optical density of test serum samples to the mean optical density of control serum samples $+ 3 \times$ the SD of the control serum samples was ≥ 1 . The figure represents antibody titers (expressed as a ratio) of the positive serum samples against each region. The table shows the percentage prevalence of positive serum samples reactive to different regions of MSP3, in terms of IgG isotype.

MSP3f were the most prevalent and were predominantly of cytophilic subclasses. Among the cytophilic isotypes, IgG3 reactivity was found to be predominant against MSP3b, MSP3c, and MSP3d. By contrast, IgG1 reactivity against MSP3f was stronger and more prevalent than that against IgG3. This suggests that the antibody response elicited to any region of MSP3 was not dependent on a response to other regions.

It had been observed earlier that the cytophilic IgG response plays an important role in protection against malaria [8–11]. We further addressed the relationship between clinical protection that had been monitored on a daily basis and the pattern of isotype responses observed against each peptide. In the present study, protection was defined as the absence of any clinical malaria attack during the 2 years after plasma samples were obtained. Higher IgG3 titers against MSP3b, MSP3c, and MSP3d were observed among protected, compared with nonprotected, subjects. An association between the levels of IgG3 antibodies directed to MSP3b and MSP3d and protection from occurrence of malaria attack ($P = .037$ and $.057$, respectively) was observed. In the case of MSP3c, this association did not reach statistical significance; however, levels of anti-MSP3c IgG3 antibodies were twice as high in individuals who did not develop malaria, compared with those who did. The association between levels of IgG1 and protection against malaria attack was observed to be significant for MSP3d ($P = .025$), and a similar trend was observed for MSP3b ($P = .328$), but not for MSP3c. Neither IgG1 nor IgG3 responses to MSP3f were found to be associated with protection. IgG2 and IgG4 antibody responses against different regions of MSP3 were detected only at low levels and were not found to be associated with protection.

In a further step, a multivariate stepwise regression analysis was performed to control for age, by use of dichotomous variables of both antibody response (responders or nonresponders) and occurrence of malaria attack (protected or nonprotected). A significant association of protection with IgG3 antipeptide responses was observed against 3 of 6 peptides—MSP3b ($F = 4.98$, $P = .025$), MSP3c ($F = 3.02$, $P = .082$), and MSP3d ($F = 6.57$, $P = .01$)—but not against the other 3.

Inhibition of parasite growth by naturally occurring antibodies against MSP3b, MSP3c, and MSP3d in functional in vitro ADCl assays. To assess the function of naturally occurring human antibodies to different regions of MSP3 in ADCl assays, each affinity-purified antibody was adjusted to a concentration that yielded the same reactivity to the native parasite protein. Results (figure 4) showed that the level of parasite inhibition elicited by antibodies against the recombinant proteins MSP3NT and MSP3CT were comparable to that observed for the pool of African IgG (PIAG), which was used elsewhere for a passive-transfer experiment in humans [6].

Anti-MSP3b, -MSP3c, and -MSP3d affinity-purified antibodies were found to exert a strong monocyte-mediated antipara-

sitic activity in ADCl that was comparable to that of antibodies against MSP3CT and PIAG, whereas anti-MSP3a and -MSP3f antibodies were not found to have parasite inhibitory activity (figure 4). Anti-MSP3e antibodies showed only marginal antiparasite activity that was slightly higher than the threshold level of significance. Results were reproducible among 4 independent ADCl assays. At the concentrations used, none of the above-mentioned antibodies showed the direct inhibition of parasite growth.

Strong reduction of *P. falciparum* parasitemia by anti-MSP3b and anti-MSP3d antibodies in a humanized mouse model. The observation from the in vitro ADCl assays that anti-MSP3b, -MSP3c, and -MSP3d antibodies were strongly effective at inhibiting parasite growth was further assessed in vivo by use of the *P. falciparum*-HuRBC-BXN mouse model. The value of this new mouse model for studying the in vivo effect of human antibodies and antimalarial drugs on the blood-stage growth of *P. falciparum* has been recently documented [16, 17]. We chose to study antibodies to MSP3d and MSP3f, which were positive and negative in ADCl, respectively, compared with anti-MSP3b antibodies, which we used as positive controls, whose antiparasitic effect has already been demonstrated [16].

As seen in figure 5, the level of parasitemia increased and reached a plateau over the next 3 weeks. The injection of anti-MSP3f antibodies with human monocytes did not affect parasite growth, in agreement with the results of the in vitro ADCl assays. In the other 2 mice, the injection of human monocytes alone on day 22 did not affect parasite growth, in keeping with earlier observations [16]. The injection of affinity-purified anti-MSP3b or -MSP3d human antibodies on day 23 resulted in a sharp decrease in parasitemia. The passive transfer of anti-MSP3b antibodies resulted in the clearance of parasites. The passive transfer of anti-MSP3d antibodies resulted in a decrease of parasitemia >95% (figure 5). Thus, results from the in vivo passive transfer in this mouse model confirmed in vitro results and further validated the functional antiparasite activity of naturally occurring antibodies against the 70-aa region covered by peptides MSP3b and MSP3d.

T cell responses against MSP3 peptides in malaria-exposed individuals. T lymphocyte responses could be studied against only 3 (MSP3a, MSP3b, and MSP3c) of 6 C-terminal peptides in inhabitants from Dielmo, Senegal, because of practical limitations in field. The proliferative response, which was determined by use of peripheral blood lymphocytes from 61 individuals (age range, 1–84 years; mean age, 27.34 years) showed that the prevalence of T helper cell responders was 16.4% against MSP3a, 28% against MSP3b, and 21.3% against MSP3c, respectively. IFN- γ secretion, which was monitored in 31 of these individuals, showed that the prevalence of IFN- γ responders was 42% against MSP3a, 55% against MSP3b, and 61.3% against MSP3c. These results indicate that each of the

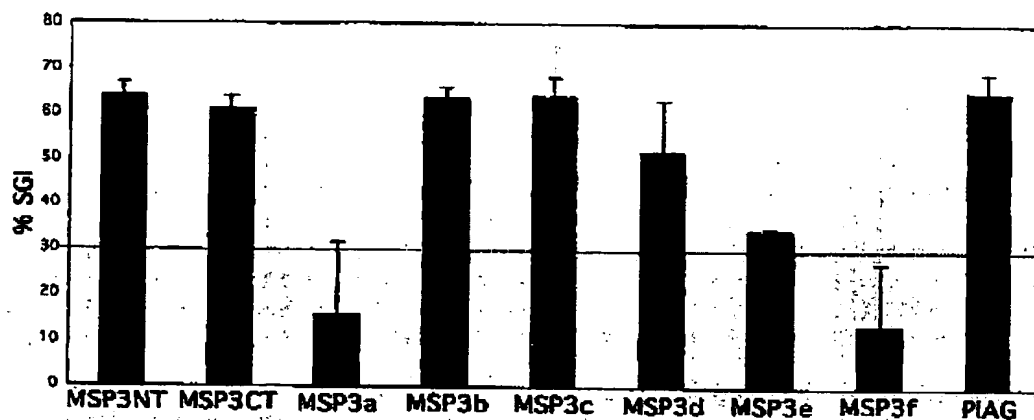


Figure 4. Effect of affinity-purified human antibodies on parasite growth in antibody-dependent cellular inhibition assay. The histograms represent mean values of the percentage of the specific growth inhibition index (SGI; as explained in the text) from 2 independent experiments \pm SE; values $>30\%$ are significant. PIAG, positive control IgG from the pool of serum samples from adults from Ivory Coast used for passive-transfer experiments in humans [6].

3 MSP3 peptides tested defines at least 1 T cell epitope. In addition, IFN- γ secretion results suggested that at least some of the responding cells belonged to the Th1-like type.

DISCUSSION

In the search for candidates for a malaria vaccine, we focused our studies on antigens targeted by the most potent immunity—that acquired over the years by individuals living in hyperendemic areas. We have described that this preimmunization is mediated by IgG that is active through an indirect mechanism, which implicates monocytes. We then used ADCI to identify

MSP3 as a target of protective IgG [12]. The present study was aimed at characterizing antigens within the conserved C terminus of MSP3 and evaluating the function and biological effects of the corresponding antibodies.

Indeed, the C-terminal half of the molecule, starting from the third heptad repeat, is highly conserved in the different isolates tested so far [14, 15], whereas the N-terminal half of MSP3 shows an overall dimorphism (3D7-like and K1-like) [14, 15]. Therefore, we decided to focus on the C-terminal region, because part of it (DG210; figure 1) was identified to be a target of protective human antibodies in our initial study

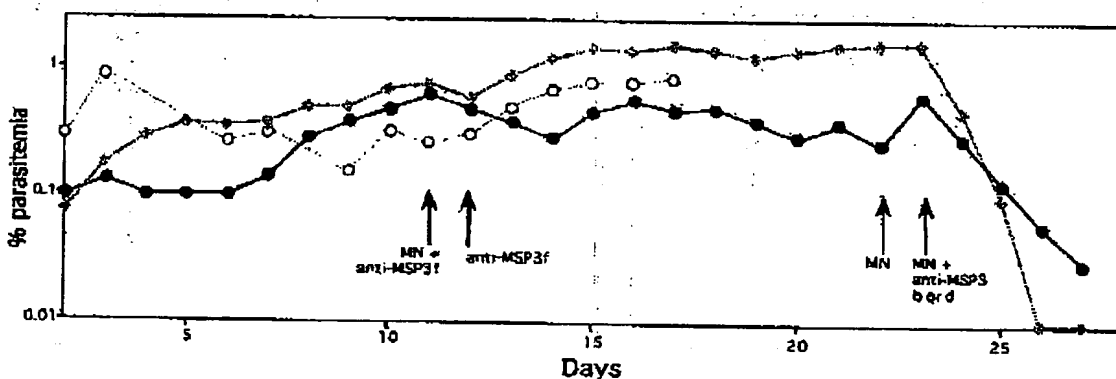


Figure 5. In vivo transfer of affinity-purified human anti-merozoite surface protein 3 (MSP3) antibodies, together with human peripheral blood monocytes in *P. falciparum*-human red blood cell-Beige-Xid-Nude mice. The curves show the course of parasitemia as determined by microscopic examination of thin blood smears from mice injected with anti-MSP3b antibodies (gray diamonds), anti-MSP3d antibodies (black circles), or control anti-MSP3f antibodies that were ineffective in the antibody-dependent cellular inhibition assay (white circles). Arrows, days at which injections were made, first of human monocytes (MNs) and then followed by monocytes together with anti-MSP3 antibodies (200 μ L; immunofluorescence assay titer, 1:200).

[12] and because antigen conservation is a critical criterion for the successful development of a malaria vaccine.

Using 6 overlapping synthetic peptides covering the conserved C-terminal half of MSP3, we have shown that antibody patterns to each region differ markedly in terms of prevalence, titer, isotype distribution, association with clinical protection, and antiparasitic activity in vitro and in vivo. Antibody titers against MSP3a and MSP3e were lower than those of the remaining 4 peptides. Responses to MSP3b, MSP3c, MSP3d, and MSP3f were mostly of cytophilic IgG subclasses—predominantly of IgG1 isotype against MSP3f and IgG3 isotype against the others. A similar difference in subclass response to distinct regions of a single protein has been reported for MSP1 [26]. These observations suggest that IgG class switching involved during the maturation of the antibody response toward different regions of the MSP3 C terminal is regulated independently. The factors that regulate the maturation of antibodies are not well understood but would be influenced by the nature of the antigen in conjunction with contact-dependent signals from T cells, particularly the cytokines they secrete [27]. Recent observations, however, have suggested that the nature of the malaria antigen might be the major factor that determines antibody subclass [28], which seems to be the case in our study.

The availability of very detailed clinical information, which is a major characteristic of the setup in the village of Dielmo, Senegal, led us to address subclass patterns in relation to protection against the occurrence of malaria. Taking into account the confounding effect of age, we observed that IgG3 responses to MSP3b, MSP3c, and MSP3d were significantly associated with protection. These results are in agreement with those of independent studies that involved larger sample sizes [29] (C. Oeury, C.R., J.L. Pérignon, C. Müller-Graf, A. Tall, C. Rogier, J.F. Trape, and P.D., unpublished data), which have shown an association between the IgG3 response against MSP3b and protection from malaria. For other merozoite surface vaccine candidates, a skewing toward the IgG3 antibody response has been reported for MSP2 in various ethnic groups and under different conditions of malaria transmission [30, 31]; this could be correlated with clinical immunity to malaria [32]. Similarly, the antibody response to the polymorphic block 2 region of MSP1, which has been identified as a target of immunity to clinical malaria, is also skewed toward the IgG3 subclass [33]. However, at least in the latter case, the mechanism of action of these antibodies remains elusive, because it is generally assumed that biologically active anti-MSP1 antibodies are directed to the C-terminal part of the antigen [34].

In contrast, in the present study, the use of functional in vitro ADCI assays provided information about the antiparasitic, biological activity of antibodies toward various regions. Because they were performed under conditions that allowed comparisons, they demonstrated critical differences in antibodies that

target different regions of MSP3. It is of interest that very different approaches led to similar conclusions—that is, the in vitro ADCI assays pointed to the importance of exactly the same peptides (MSP3b, MSP3c, and MSP3d) as those indicated by the immunoepidemiological studies. The reasons for this lack of effect of antibodies to MSP3a and MSP3f remains to be investigated. In the case of MSP3f, it is possible that antibodies might not access this epitope on the merozoite surface, because the leucine-zipper domain forms coiled-coil interactions with other molecules [13, 14].

The reliability of in vitro findings could also be confirmed under in vivo conditions [16]. On passive transfer in *P. falciparum*-infected mice grafted with human monocytes and with long-lasting stable parasitemia, anti-MSP3b and -MSP3d antibodies were found to be effective in reducing the *P. falciparum* parasite load.

The vaccine potential of MSP3 was recently confirmed by the protection elicited against *P. falciparum* challenge in *Aotus nan-cymai* monkeys immunized with full-length MSP3 in Freund's adjuvant [35]. This observation is in agreement with our epidemiological and biological findings. However, the present study has provided additional information derived from the analysis of human immune responses for the design of future vaccine constructs. Indeed, the N terminal of MSP3, although able to induce antibody with functional activity in ADCI, is of debatable value, because of its polymorphism. Furthermore, its inclusion could divert the immune response away from the important conserved region. Within the C-terminal part, the region MSP3e-f was also found to be less valuable, because of the low prevalence and low levels of antibody response to MSP3e and anti-MSP3f antibodies devoid of biological effect. Each of the 3 peptides (MSP3a, MSP3b, and MSP3c) investigated proved to define a non-cross-reactive T cell epitope for populations in endemic areas. Recent vaccine trials performed using the construct defined in the present study confirmed this finding and designated the peptide MSP3d as an additional T cell-epitopic region (R. Audran, M. Cachat, F. Lurati, S. Soe, O. Leroy, G.C., P.D., and R. Spertini, unpublished data).

In summary, the results of immunoepidemiological studies and functional assays led us to define a 70-aa region of the MSP3 molecule. We found that antibodies with antiparasitic effect develop against this region, which covers MSP3b-MSP3d, in humans who have been naturally exposed to malaria. This information is of practical value for future clinical trials for the rational design of subunit vaccine constructs derived from MSP3.

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ANNEX III

Parasite Immunology, 2004, 26, 265–272

Natural antibody response to *Plasmodium falciparum* Exp-1, MSP-3 and GLURP long synthetic peptides and association with protection

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SUMMARY

A longitudinal study was undertaken in Burkina Faso among 293 children aged 6 months to 9 years in order to determine the correlation between an antibody response to several individual malarial antigens and malarial infection. It was found that the presence of a positive antibody response at the beginning of the rainy season to three long synthetic peptides corresponding to *Plasmodium falciparum* Exp-1 101–162, MSP-3 154–249 and GLURP 801–920 but not to CSP 274–375 correlated with a statistically significant decrease in malarial infection during the ongoing transmission season. The simultaneous presence of an antibody response to more than one antigen is indicative of a lower frequency of malarial infection. This gives scientific credibility to the notion that a successful malaria vaccine should contain multiple antigens.

Keywords antibody, longitudinal study, malaria, *Plasmodium falciparum*, synthetic peptide, vaccine

Abbreviations: Ab, antibody; BSA, bovine serum albumin; CSP, circumsporozoite protein; GLURP, glutamate-rich protein; IgG, immunoglobulins; MSP-3, merozoite surface protein-3; *Pf*, *Plasmodium falciparum*; *Pf*Exp-1, *Plasmodium falciparum* exported protein-1

INTRODUCTION

The asexual blood stages of the *Plasmodium falciparum* parasite are responsible for the clinical symptoms of malarial infections, and people living in areas of high *P. falciparum* endemicity are exposed to repeated malarial infections and gradually develop clinical protection against the disease over a period of several years (1). This acquired immunity is strong, although incomplete and is non-sterilizing (2,3). These observations support the feasibility of a malaria vaccine. Passive transfer experiments, in which immunoglobulin G (IgG) was transferred from clinically immune individuals to infected patients, have shown that antibodies are an important component of acquired immunity to asexual blood stages of *P. falciparum* (4,5). Identification of the malarial antigenic targets of potentially protective antibody responses following natural infection could help with the understanding of the host–parasite relationship and provide information for the selection of candidate antigens for malaria vaccines.

Several *P. falciparum* asexual blood stage antigens have been examined in seroepidemiological surveys conducted in malaria-endemic areas and were recognized by the immune responses of individuals exposed to natural infection. These antigens include merozoite surface protein-1 (MSP-1) (6,7), MSP-4 (8), ring-infected erythrocyte surface antigen (RESA) (9), rhoptry-associated proteins-1 (RAP-1) (10), erythrocyte binding antigen-175 (EBA-175) (11), *P. falciparum* erythrocyte membrane protein-1 (*Pf*EMP-1) (12–14), glutamate-rich protein (GLURP) (15,16) and merozoite surface protein-3 (MSP-3). In some cases, positive associations were observed between the antibody responses and clinical protection against malarial infection, indicating a protective role for antibodies to these antigens (6,13–17).

Plasmodium falciparum exported protein-1 (*Pf*Exp-1) is a vesicular protein, which is believed to play a role in the trafficking of parasite proteins (18). *Pf*Exp-1 is a highly

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conserved protein, suggesting that it either has an important function in the parasite (18,19) or that it is not under immune pressure. *PfExp-1* is expressed as a 23 kDa protein in the pre-erythrocytic (20) and asexual blood stages (21) of the parasite. This integral membrane protein is found in the membrane of the parasitophorous vacuole and in vesicles within the host cell cytoplasm (22–24). Studies with the murine homologue of *PfExp-1* indicate that this protein is capable of inducing a protective T-cell immunity in mice against lethal challenge with *P. yoelii* (25) and monoclonal antibodies to *P. yoelii* Exp-1 can inhibit parasite growth (26). Anti-*PfExp-1* antibodies have been detected in people living in different regions where malaria is endemic (27). It has further been shown that most of the detectable human antibodies are targeted at the C-terminal sequence 101–162 of *PfExp-1* (27). In a preliminary cross-sectional study, 150 serum samples of Burkinabe children, aged between 0.5 and 6 years, were tested by ELISA with different synthetic peptides, namely: the C-terminal segment of circumsporozoite protein (CSP), the central repeat NANP of CSP and *PfExp-1* 73–162. A statistically significant difference in the antibody response between asymptomatic and mild or severe malaria cases was found only with the long synthetic polypeptide (LSP) *PfExp-1* 73–162 (28). To confirm and extend these results, a longitudinal study was designed to determine the association of specific antibody responses and malaria episodes in a population of children living in Burkina Faso, where malaria is highly endemic and seasonal. Other antigens, already associated with protection, were included in the study to provide further validity of the results obtained. It was found that the preliminary results linking the antibody response against Exp-1 to protection were confirmed and that the response to more than one antigen at the beginning of the transmission season was predictive of a greater resistance to malaria.

MATERIALS AND METHODS

Study area

The study was conducted in Balonguen, a village located on the Mossi Plateau in Burkina Faso. It is predominantly a subsistence farming community with a population of about 1355. The climate is characteristic of areas of Sudanese savannah, with one rainy season from June to October and a dry season from November to May. Malaria transmission is seasonal, but is highest during or immediately after the rainy season (high-transmission season) and lowest during the dry season (low-transmission season). Entomological studies performed during the month of September 2001, at the peak of transmission, measured 129 infective bites per person per month (EIR) for the area near the village of

Balouguen (Issa Nebié personal communication). For the month of March, the measured EIR for the area was near zero (Issa Nebié, personal communication).

Study population and clinical surveillance

The study population consisted of 326 children, 6 months to 9 years of age, of whom 46% were males and 54% were females. The cohort included between 12 and 64 children at each year of age. Informed parental consent was obtained after thorough explanation of all procedures involved in the study, which was approved by the Ministry of Health of Burkina Faso. The study began in May 2001 with a cross-sectional survey and ended in November with a final cross-sectional survey. A third cross-sectional survey was performed in the middle of the rainy season, in August.

In the course of this longitudinal study, the cohort of children was monitored clinically and parasitologically by the field assistants of the Centre National de Recherche et de Formation sur le Paludisme (CNRFP), who were resident in the village. Each child was visited every 2 days; during each visit, information regarding the health status was recorded on a standard questionnaire and systematic measurement of axillary temperatures was determined with a mercury thermometer. Blood films for the detection of parasitaemia were made from children with temperatures of $\geq 37.5^{\circ}\text{C}$. Parents were also instructed to bring sick children to the field assistants outside the scheduled visits, for recording of temperature, blood parasitaemia and for medication. Any child with fever was immediately treated with chloroquine, according to the recommendation of the National Malaria Control Programme. Individuals were considered to have a clinical malaria episode only (i) if they had a measured temperature $\geq 37.5^{\circ}\text{C}$ and (ii) if they had parasitaemia of ≥ 5000 parasites/ μL . This level of parasitaemia has previously been used as a criterion for clinical malaria in another epidemiological study (29). In the case of several episodes of malaria occurring in the same individual, these were considered to constitute discrete episodes only if they were separated by at least 3 weeks.

The data on the association of protection with an antibody response included in this report were from samples obtained from 293 children for whom clinical and parasitological data were available for the duration of the 7-month follow-up period and from whom serum samples were obtained in May 2001 (see below). The composition of this group was not significantly different from the full cohort of the first survey in May 2001 (T1) ($n = 326$) (data not shown).

Parasitological diagnosis

Thick and thin blood films were air-dried; thin blood films were fixed with methanol and both stained with Giemsa 3%.

One hundred high power fields were examined and the number of malaria parasites of each species and stage recorded. The number of parasites per microlitre of blood was calculated assuming a fixed white cell count of 8000 per microlitre.

Sample collection

Blood samples were obtained from the cohort on three occasions. The first samples were collected in May 2001 (T1), just before the onset of the rainy season, which corresponds to the high malaria transmission season. The second samples were collected in August 2001 (T2) during the peak of the high transmission season and the third samples in November 2001 (T3) at the end of the high transmission season. 500 µL to 1 mL of blood from each donor were drawn into microtubes. Serum samples were stored at -20°C until use. Negative control serum samples were obtained from 16 healthy Swiss adults, who had never lived in a malaria-endemic area. A pool of plasma samples obtained from adults living in Burkina Faso and selected for high antibody reactivity to the LSP PfExp-1 101-162 were used as positive control.

Peptides

All LSP used in this study were chemically synthesized using solid phase F-moc chemistry, as described (30,31). The crude LSP were purified by a combination of size exclusion chromatography (Sephadex G25, Pharmacia, Sweden) and RP-HPLC (Phenomenex, Rancho Palos Verdes, USA) using a 10-50% CH₃CN gradient in 0.1% TFA/H₂O. The degree of purity of the LSP was analysed by RP-HPLC and mass spectrometry. Stock solutions of LSP (10 or 20 mg/mL) in DMSO (Sigma, MO, USA) were stored at -20°C. LSP are PfExp-1 101-162 (SWISS-PROT database primary accession number P04926), MSP-3 154-249 (TrEMBL database primary accession number Q8IJ55), GLURP 801-920 (TrEMBL database primary accession number Q25860) and CSP 274-375 (SWISS-PROT database primary accession number P19597).

Enzyme-linked immunosorbent assay (ELISA)

Serum samples were evaluated in duplicate by ELISA, at a dilution of 1 : 200, for the presence of IgG antibodies specific for the LSPs as described (27). The antibody ratio (Ab ratio) of the serum samples was calculated as the ratio of the optical density (OD) value of the test sample divided by the mean plus 3 standard deviations (SD) of the 16 Swiss negative control serum samples. A donor was considered positive for a LSP when the Ab ratio for this LSP was greater than one.

The positive standard was a pool of serum samples from adult Burkinabe immune to malaria. Titration of the standard was performed against each LSP in each experiment to control for inter-experimental variation.

Data analysis

Data were entered using Epiinfo Version 6 and Excel version 2000. The statistical analysis was performed with STATA version 7.0. Children with antibody ratio > 1 were considered seropositive and those with antibody ratio ≤ 1 were considered seronegative. A malaria episode was defined as fever (temperature ≥ 37.5°C) with presence of at least 5000 asexual blood stages of *P. falciparum*. Malaria episodes were grouped into two categories: no malaria episode and one or more malaria episodes. The Wilcoxon signed-rank test and the Mann-Whitney rank-sum test were used to compare the antibody ratio between the groups for paired and unpaired data, respectively. The χ^2 test was used to compare proportions of children with at least one malaria episode between seropositive and seronegative responders. Multivariate analysis was performed by fitting a logistic regression, taking into account the effect of age and sex, to measure the association between serological status at each survey period and malaria episodes in the period of high malaria transmission. Differences were considered statistically significant if $P < 0.05$. Kaplan-Meier curves were plotted to compare malaria attacks in seronegative and seropositive children for the 3 LSP.

RESULTS

Seroprevalence and age

Three cross-sectional surveys were performed in May, August and November 2001 on the same cohort of 326 children. In all three cross-sectional studies, 87% of the evaluated children displayed IgG antibodies to at least one LSP, Exp-1 101-162, MSP-3 154-249 or GLURP 801-920. For the May survey, the proportion of children with positive Ab ratio for LSP Exp-1 101-162 and GLURP 801-920 was 30-35% at 0-5-3 years of age, 60-70% at 3-4 years of age and around 80% for children 5-9 years old. For LSP MSP-3 154-249 the percentage of seropositive children was 31-5% at 0-5-3 years of age, 45% at 3-4 years of age, 60% at 5-7 years of age and 83-6% at 8-9 years. For CSP LSP the prevalence was around 20% at 0-5-4 years of age, 27% at 5-7 years of age and 43-6% at 8-9 years (Table 1). During the May survey, the proportion of children with positive parasitaemia was 44%. Antibody response to the 3 LSP was significantly higher in children with positive parasitaemia than in children with negative parasitaemia. The mean antibody ratios were 3.0.

Table 1 Proportion of children with positive antibody response to the evaluated LSP at the first cross-sectional survey

Age group (years)	Percentage (n/total) of children with positive antibody responses			
	P/Exp-1 101-162	MSP-3 154-249	GLURP 801-920	CSP 274-375
0-5-3 years	57.4 (31/54)	31.5 (17/54)	35.2 (19/54)	20.0 (8/40)
3-4 years	73.2 (52/71)	45.1 (32/71)	60.6 (43/71)	23.5 (12/51)
5-7 years	81.3 (83/102)	60.0 (61/102)	82.3 (84/102)	27.3 (21/77)
8-9 years	85.1 (57/67)	83.6 (56/67)	80.6 (54/67)	43.6 (24/55)
Pearson's χ^2	15.7	38.17	44.1	8.1
P-value	$P = 0.001$	$P < 0.001$	$P < 0.001$	$P = 0.044$

n, number of children with positive antibody responses

Table 2 Proportion of children with a single or multiple antibody response to the 3 LSP and their mean number of malaria episodes

	Antibody response to			
	0 LSP	1 LSP	2 LSP	3 LSP
Percentage (n/total) of children with Ab ratio > 1	13 (38/293)	17.1 (50/293)	25.9 (76/293)	44 (129/293)
Mean number of malaria episodes	1.01	0.49	0.29	0.20
		$P < 0.001^a$		
Odds Ratio (risk of developing one or more episodes of malaria adjusted for age)	1.0	0.29, $P < 0.001$	0.11, $P < 0.001$	0.007, $P < 0.001$

^aKruskal-Wallis test for the difference in number of malaria episodes between children with and without antibody response against at least one LSP.

2.3 and 4.4 in the former group of children vs. 2.1, 1.2 and 3.1 in the latter group of children, respectively, for P/Exp-1, MSP-3 and GLURP.

LSP response in relation to clinical protection against malaria

The number of children who were involved in the May survey and followed up during the longitudinal survey was 293. During the follow-up period 299 episodes of fever + parasitaemia were observed, and 144 of these events met our definition of a malaria episode (fever + 5000 asexual forms/ μ L).

In order to establish whether a pre-existing antibody response to one of the LSP could influence the number of malaria episodes, the antibody response to the LSP was compared at the beginning of the rainy season, May 2001, with the number of malaria episodes reported during the longitudinal study. For LSP P/Exp-1 101-162, it could be observed that children without any episode had a significantly higher antibody response than children with 1, 2 or 3-4 reported malaria episodes (Mann-Whitney rank-sum test: $P < 0.001$, $P = 0.0053$ and $P < 0.001$, respectively; Figure 1a). Similar results were obtained for LSP MSP-3 154-249 ($P = 0.0055$, $P < 0.001$ and $P < 0.001$, respectively; Figure 1b) and LSP GLURP 801-920 ($P < 0.001$, $P < 0.001$

and $P < 0.001$, respectively; Figure 1c). Kaplan-Meier estimates were plotted to compare the probability of getting the first malaria episode between seropositive and seronegative children for the 3 LSP (Figure 2a,b,c) at the beginning of malaria transmission season, May 2001. The probability of developing clinical malaria was significantly lower in children who were seropositive for P/Exp-1 101-162, MSP-3 154-249 and GLURP 801-920 at, respectively, 0.07, 0.09 and 0.04. In seronegative children the chances of developing a malaria episode were 0.3, 0.33 and 0.37 (in all cases log-rank test P -value was less than 0.001). In addition, since children presented an antibody response to more than one antigen, it was asked if this correlated with a lower malaria risk. As seen in Table 2, lower malaria episodes were detected in children with a response to more than one antigen. However, because the antibody ratios to the LSP correlated significantly with age (Spearman's rank correlation test: $P < 0.001$ for Exp-1, MSP-3 and GLURP; data not shown), we analysed the number of malaria episodes and serological status of children using a logistic regression model (Table 3). The presence of antibodies to LSP Exp-1, MSP-3 or GLURP but not CSP was correlated with a lower incidence of malaria episodes observed in the following malaria season. Having parasitaemia before the high transmission season did not appear to affect the association between responding

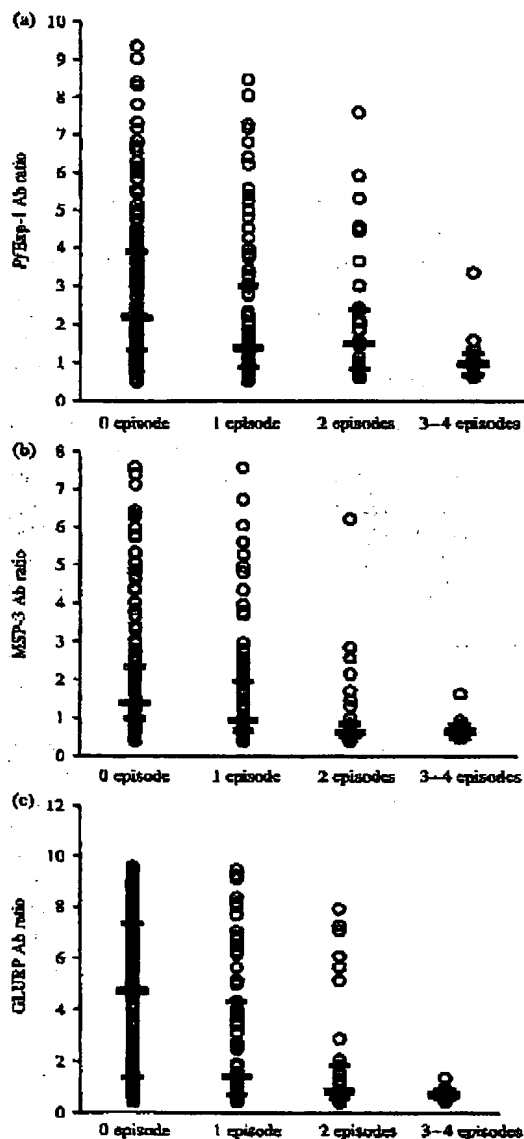


Figure 1 Associations between antibody responses to the LSP and number of malaria episodes. Children were clinically and parasitologically monitored from May to November 2001. The serum samples were evaluated by ELISA for the recognition of LSP P/Exp-1 101–162 (a), MSP-3 154–249 (b) or GLURP 801–920 (c). The serum samples ($n = 293$) were collected at the end of the dry season in May 2001 (T1). The major horizontal line indicates the median of the antibody ratios and the minor horizontal lines the 25th and 75th percentiles. The number (n) of children was as follows: 0 episodes, $n = 165$; 1 episode, $n = 84$; 2 episodes, $n = 33$ and 3–4 episodes, $n = 12$.

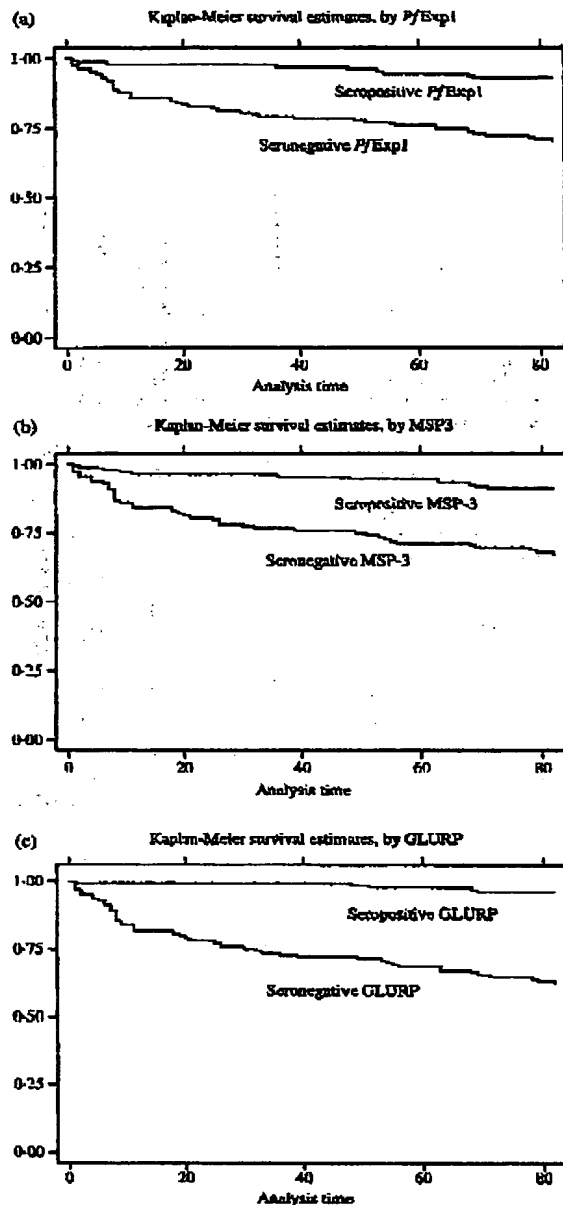


Figure 2 Kaplan-Meier curves for the occurrence of the first malaria episode in children seropositive and seronegative for the 3 LSP. Children were clinically and parasitologically monitored from May to November 2001. The serum samples were evaluated by ELISA for the recognition of LSP P/Exp-1 101–162 (a), MSP-3 154–249 (b) or GLURP 801–920 (c).

Table 3 Association between serological status to the evaluated LSP during cross-sectional survey of May (T1) and malaria episodes

LSP	Serological status	Mean number of malaria episodes	Percentage (n/total)	Odds ratio ^a (95% c.i. ^b) P-value
<i>PfExp</i> -1 101–162	Seronegative	0.68	29.4 (48/163)	0.23 (0.10, 0.51)
	Seropositive	0.25	6.9 (9/130)	$P < 0.001$
MSP-3 154–249	Seronegative	0.72	33.1 (42/127)	0.32 (0.16, 0.64)
	Seropositive	0.31	9.0 (15/166)	$P = 0.001$
GLURP 801–920	Seronegative	0.79	37.2 (51/137)	0.11 (0.04, 0.28)
	Seropositive	0.23	3.9 (6/156)	$P < 0.001$
CSP 274–375	Seronegative	0.56	24.1 (38/158)	0.47 (0.18, 1.18)
	Seropositive	0.30	10.8 (7/65)	$P = 0.11$

n, number of children with one or more malaria episodes.

^aOR adjusted for age and sex using logistic regression; ^bc.i.: confidence interval.

to one or more LSP and the risk of experiencing a malaria episode. A logistic regression model including the 3 LSP suggest that the 3 LSP were comparable in reducing malaria attacks with slightly higher protective effects for *Exp*-1 and GLURP.

DISCUSSION

The long synthetic polypeptides *PfExp*-1 101–162, MSP-3 154–249 and GLURP 801–920, representing antigenic fragments of three asexual blood stage antigens of *P. falciparum* parasite, were used to study the acquisition and evolution of antibodies during natural malaria infection in children living in a hyperendemic area in Burkina Faso. The results demonstrated that there was a high prevalence (up to 87%) of serum IgG to at least one of the three LSP among the evaluated Burkinabe children, and this prevalence increased with age. The levels of the antibody responses against these tested LSP also increased with the age of the children, and were positively associated with a reduced number of clinical malaria episodes. The results obtained for LSP GLURP 801–920 are in complete accordance with two other longitudinal studies where a recombinant GLURP protein covering the sequence 489–1271 (17) and the same LSP GLURP 801–920 (32) were used. This accordance with already published studies validates the correlation described here between protection and the level of antibody response to *PfExp*-1 101–162 and MSP-3 154–249.

The prevalence of positive response to the 3 LSP remained stable over the 3 cross-sectional surveys of the transmission season (data not shown).

The lack of antibody response to any of the LSP in 13% of the evaluated children is most likely related to their young age (mean age 3.1 years) and lower parasite exposure compared to children that responded to 2 or 3 LSP (mean age 4.9 and 5.9 years, respectively). In fact, previous sero-epidemiological studies conducted with monozygous African

twin pairs have shown that genetic factors do not play a major role in determining antibody responsiveness to various malaria antigens (33,34). It is also possible that the sequence polymorphism of the R2-repeat region of GLURP among different alleles of parasite could induce antibodies with other specificities than the one present in our sequence 801–920 (35). This remark is not applicable for LSP MSP-3 154–249, where the C-terminal fragment of MSP-3 has been shown to be highly conserved in laboratory clones and field isolates (36). Similar consideration should apply to the *PfExp*-1 molecule. The weak polymorphism of the whole sequence of *PfExp*-1 observed among laboratory strains (18,19) needs to be confirmed with the genotyping of *P. falciparum* parasites isolated from donors living in the same endemic area of this longitudinal study in Burkina Faso. Determination of the presence and frequency of eventual point mutations among field isolates and comparison with the humoral immune response against LSP *PfExp*-1 101–162 may explain the absence of response in the serum samples of certain children.

Recent studies have shown that GLURP (15,37) and MSP-3 (38) contain B-cell epitopes that are targeted by cytophilic IgG such as IgG1 and IgG3, and in conjunction with blood mononuclear cells via their FcγRII receptors trigger the release of killing factors such as TNF-α (39). This mechanism is called antibody-dependent cellular inhibition (ADCI) (40). Determination of the isotype(s) of the IgG against LSP *PfExp*-1 101–162 and the study of an eventual association with protection among children and adults from endemic areas could explain the mechanism of action of anti-*PfExp*-1 101–162 antibodies, and it is expected to be important for the choice of adjuvant and for optimization of the protocol of immunization for future clinical trials.

It should also be pointed out that the association between protection and antibodies specific for a given B-cell epitope does not indicate that the antibody response to the whole protein is protective (41), and the use of LSP covering a

whole protein domain with defined conformation is of interest to discriminate between protective and immunodominant epitopes. The results of this longitudinal study with three different and unrelated LSP point toward the design of a subunit malaria vaccine. In fact, it seems the level of the antibody response to a given antigen and the number of antigens simultaneously targeted by antibody responses are important for the protection. The strong association between the pairwise antibody response to the LSP PfExp-1 101-162, MSP-3 154-249 and GLURP 801-920 indicates that protected children are the ones that can positively respond to different asexual blood stage antigens. Since a few children without antibody to any of the 3 LSP remained free of clinical malaria episodes during the longitudinal survey, other B-cell epitopes on the evaluated antigen or other malaria asexual blood or pre-erythrocytic antigens must also be targeted by protective antibodies or T-cell response. Therefore, further studies with other B-cell epitopes of the evaluated antigens or with other asexual blood stage antigens could help us to shape the repertoire of the targets of the protective immunity.

To conclude, this study demonstrates in children a positive association between their level of IgG specific for PfExp-1 101-162, GLURP 801-920 and MSP-3 154-249 before the raining season and subsequent protection from clinical malaria episodes. The predictive value of such an analysis needs to be confirmed in other settings. In addition, these results justify further studies of the PfExp-1 protein to understand its mechanism and contribution to the acquired protective immunity and place PfExp-1 in the rank of a malaria vaccine candidate together with the MSP-3 and GLURP molecules.

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